1st Faculty of Medicine, Charles University in Prague

Doctoral Thesis (short version)

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Proteomic analysis of liver iron overload

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1. Introduction

Iron is an essential element for virtually all living organisms. It is required as a cofactor for a multitude of proteins of diverse biological function. Iron plays a central role in oxygen transport by hemoglobin, in oxidation-reduction reactions, in cellular proliferation, and in many other biochemical pathways and physiological processes.

The dark side of the metal is, that in excess, it can be toxic. This is due to the deleterious effects of reactive oxygen species (ROS), such as the hydroxyl radical ($OH^{\bullet}$), which is produced via the Fenton reaction. The highly reactive hydroxyl radical is able to cause oxidative damage to DNA, proteins and biological membranes. These oxidative reactions can result in impairment of cellular functions, cellular damage and apoptosis. Because of this dual nature of iron, the concentration of the metal in mammalian body must be kept within defined limits and there are precise mechanisms governing the regulation of iron homeostasis. In humans there is no active mechanism for the excretion of iron, so the amount of iron in the body is determined by the regulation of iron absorption in the proximal small intestine. Consequently, iron levels are tightly regulated by specialized proteins, that transport and store iron in a soluble and non-toxic form.

Disruption of iron metabolism can lead to iron deficiency or iron overload. Iron overload is involved in the pathogenesis of many human diseases. Among all iron loading disorders, hereditary hemochromatosis and transfusion-dependent iron overload, are of utmost concern, considering their epidemiological impact, extent of iron burden, and risk for iron-related morbidity and mortality. Surplus iron in mammalian body accumulates in parenchymal organs, namely, in the liver, untreated iron overload leads to

liver fibrosis, cirrhosis, and cancer (Pietrangelo, 2004).

Hereditary hemochromatosis (HH) type I is a genetic iron overload disease, associated with mutations in the *HFE* gene, with clinical consequences that can result in liver fibrosis, cirrhosis, diabetes, heart failure, arthritis or liver cancer (Feder et al., 1996). The protein encoded by *HFE* gene is similar to MHC class I-type proteins and associates with β2-microglobulin (β2M). The majority of HH patients carry a mutation that prevents formation of a disulfide bond, impairing the physiological association of HFE with β2M. Lack of the association dramatically reduces cell surface expression of HFE (Pietrangelo, 2004). Molecular mechanism of hereditary hemochromatosis type I is unknown, despite the knowledge of underlying mutation in HFE protein.

Our knowledge of pathophysiological processes connected with or triggered by iron deposition in liver is very limited. To get a deeper insight into the molecular pathology of iron overload, we studied the effect of iron overload in liver cells with the use of proteomic techniques. In the studies presented here, influence of acute and chronic iron overload on liver cells and liver was investigated in two different models: human hepatoma HepG2 cells and C57BL/6J mice. Molecular mechanisms of genetic iron overload was studied in mouse model of hereditary hemochromatosis ($HFE^{-/-}$ mice).

2. Aims

We focused our studies of molecular pathophysiology of liver iron overload on the three central questions:

1. **What changes in protein expression induces acute iron-overload in liver cells?**
2. **What changes in protein expression triggers chronic iron overload in liver?**
3. **Are there any liver protein expression changes specific for hereditary hemochromatosis that are not caused by iron overload?**

We attempted to answer these questions with the help of proteomic methods described in the next paragraphs.

3. Methodical approach

3.1. Proteomics

The complete set of proteins present in a cell or a tissue in a given moment, including all protein modifications, interactions, localization and metabolic turnover, is called proteome. Any disturbance of protein expression, structure or localization may lead to disturbance of physiological processes and that may ultimately result in a disease. Proteomics, a relatively new set of modern techniques and approaches aims to quantitatively and qualitatively characterize all proteins present in a given moment in a given organism, tissue or cell. Expression proteomics aims to compare proteomes under various conditions.

The starting material of a proteomic analysis is usually a mixture of hundreds or rather thousands of various proteins originating from cell cultures, tissues, body fluid or other sources. The principle of most proteomic experiments is to separate these mixtures (usually by electrophoresis or chromatography) and then to identify and quantify the individual proteins by mass spectrometry. In proteomics, the most often used tool of the protein separation is two-dimensional gel electrophoresis (2-DE), which we also used in our studies.

The principle of 2-DE is two-dimensional electrophoretic separation of proteins based on two different physico-chemical properties of proteins. At first on the basis of their charge (isoelectric point) and then orthogonally on the by their molecular weight. Separated proteins are detected in 2-DE gels by staining. Routinely, up to 1500 individual protein spots can be detected in a single gel. The resulting electrophoretogram represents a specific profile of protein expression in the given tissue. The

stained gels are scanned and subjected to a quantitative image analysis. The changes in intensity/size of spots, i.e. protein expression, are accurately quantified and the protein spots with signifaicantly changed intensities are cut out from the gel, digested with trypsin, and identified by mass spectrometry.

In our project, we used the classical method of expression proteomics - combination of 2-DE with mass spectrometry. We concentrated on:

1) revealing the changes in protein expression elicited by: iron overload in cellular and animal model

2) the protein expression changes characteristic for the mouse model of hereditary hemochromatosis.

Common methodical features of all three studies included in the presented thesis are summarized in the following workflow scheme:

Manipulation of the model (changes of iron levels) → homogenisation of the sample (liver cell or liver tissue) → protein sample separation by 2-DE → staining, analysis of electrophoretograms and selection of differentially expressed protein spots → digestion by trypsin → extraction of peptides and identification by MS → verification of the results by an independent method (mRNA, Western blot).

4. Summary of results and discussion

To get a deeper insight into the molecular pathophysiology of iron overload, we studied the effect of iron overload in liver and liver cells with the use of proteomic techniques. We focused our studies on answering the three central questions that defined our specific aims (see chapter 2).

4.1. Aim One:

What changes in protein expression induces acute iron-overload in liver cells?

In mammalian body, surplus iron is deposited in parenchymal organs, mainly in the liver. Therefore, we studied changes of protein expression in HepG2 cells induced by high levels of cellular iron. HepG2 cells are derived form hepatocellular carcinoma and have most phenotypic characteristics of a standard hepatocyte, as for example: synthesis of alfa2-macroglobulin, alfa1-antitrypsin, transferrin, alfa1-antichymotrypsin, haptoglobin, ceruloplasmin, and plasminogen (Knowles et Aden, 1983). After cultivation in a standard cell cultture media with addition of 1m M Fe (in the form of $FeSO_4$) for 72 hours, these cells contained 13 times more internalized iron than control cells. Cell viability remained unafected.

With the help of 2-DE and mass spectrometry we discovered changes in expression of 21 protein spots (of 1060 protein spots) and identified 19 of them. Expression of 11 proteins was increased, 8 proteins were down-regulated as a result of acute iron overload. Some of the identified proteins have been associated with iron overload or oxidative stress previously, identification of others in the context of iron overload was

new.

Iron overload, oxidative stress and PDI

As expected, expression of L-ferritin (iron storage protein) was 10 times higher in $FeSO_4$ treated HepG2 cells. However, even the massively increased ferritin expression did not prevent oxidative stress and lipid peroxidation in the iron-loaded HepG2 as was clearly demonstrated by 20-fold increase in reactive lipid peroxides and aldehydes.

Protein disulfide-isomerase (PDI), up-regulated in this experiment, is a multifunctional protein. PDI plays important role in corrective protein folding and also in collagen production, where it serves as beta subunit of prolyl 4-hydroxylase (P4H) (Wilkinson et Gilbert, 2004). We hypothesized that increased cellular iron induced not only PDI, but also the second subunit of the P4H complex. We tested the expression of the enzymatically active α1-subunit of prolyl 4-hydroxylase at the mRNA level in control and iron-overloaded HepG2 cells. We found out that α1-subunit mRNA was markedly decreased by iron overload. Therefore, we assume that the observed up-regulation of PDI reflects intrinsic functions of PDI, independent of P4H activity, namely, rearrangement of disulfide bridges. Therefore, we propose that increased PDI concentration compensates for higher need of corrective disulfide isomerization in iron-overloaded HepG2 cells, where oxidative stress is significant.

Laminin receptor, keratins

The laminin receptor, up-regulated in this experiment, is a protein with high-affinity to laminin. Laminins are a family of extracellular matrix proteins and represent the major noncollagenous glycoprotein found in the basement membrane. Laminins and the laminin receptor are involved in

many physiological processes, such as cell attachment, cell migration, growth, and differentiation. Laminin has been recently demonstrated to induce the expression of keratin 19 in hepatoma cells in culture. This process is thought to involve the laminin receptor (Su et al., 2003). We observed increased expression of the laminin receptor as well as of keratin 19 and keratin 8 in response to iron overload. Therefore, we presume that there is a direct link of signal events connecting iron overload with increased expression of the laminin receptor and increased production of the cellular antistress protein keratin 19 in iron-loaded HepG2 cells.

Enzymes and regulatory proteins

Among the proteins whose expression was down-regulated by iron overload was the transcriptional regulator KAP-1 (KRAB-associated protein). This protein is a universal corepressor for a large family of transcription factors, the KRAB domain-containing zinc finger proteins (Friedman et al., 1996). KAP-1 exerts its corepressive function in association with various proteins, for instance, with the chromatin remodeling protein HP-1 (Ryan et al., 1999), and histone deacetylase complexes (Underhill et al. 2000). Because KAP-1 is versatile molecule engaged in several regulatory processes, the significance of its down-regulation in response to iron and oxidative stress may be very complex and remains to be elucidated.

A very surprising finding was the identification of a human homolog of anamorsin. Anamorsin is a cytokine-induced apoptosis inhibitor discovered in mice in 2004 (Shibayama et al., 2004). So far, nothing is known about the human anamorsin function and expression; we can only analogize based on the known mouse homolog. It has been demonstrated that mouse anamorsin is a critical antiapoptotic protein essential for

hematopoiesis. We presume that reduced expression of the human anamorsin homolog in response to iron and oxidative stress can signal an increased proapoptotic trend in oxidatively damaged cells. To our knowledge, our observation is the first piece of direct information provided on human anamorsin expression and its putative functions.

Dihydrolipoamide dehydrogenase-binding protein (DDBP), down-regulated by iron overload, is required for anchoring dihydrolipoamide dehydrogenase (E3) to the dihydrolipoamide transacetylase (E2) core of the pyruvate dehydrogenase complexes in mitochondria. We hypothesize that the observed decreased expression of protein DDBP in iron-overloaded HepG2 cells leads to a partial pyruvate decarboxylase deficiency and depletion of NADH with decrease of ATP production by mitochondria. This is in accordance with the disturbances of mitochondrial oxidative metabolism and decreased ATP production observed previously in cells exposed to excess iron (Bacon et al., 1993).

The expression changes of some other proteins (enolase 1 and 2, aldehyde dehydrogenase 2) are probably not related to iron metabolism, more likely these proteins respond to a general stress (Petrak et al., 2008).

In summary, by employing proteomic approaches, we identified 19 proteins differentially expressed by iron overload and the consequent oxidative stress in human hepatoma cells. To our knowledge, this was the first attempt to use modern proteomic techniques to study iron overload in mammalian cells.

The results of our study were published in American Journal of Physiology in 2006:

Petrak J, Myslivcova D, Man P, Cmejla R, Cmejlova J, Vyoral D. Proteomic analysis of iron overload in human hepatoma cells. Am J

Physiol Gastrointest Liver Physiol. 2006, 290(5): G1059-66.

4.2. AimTwo:

What changes in protein expression triggers chronic iron overload in liver?

For this set of experiments common inbred C57BL/6J mice were used as model organisms. Sixteen 3-months-old male mice were divided into two groups of eight animals and fed ad libitum with either defined standard rodent diet (C1000, Altromin, 180 ppm Fe) or the same diet supplemented with 2% carbonyl iron.

Iron overload induced by carbonyl iron-supplemented diet is known to result in a predominantly parenchymal iron deposition pattern similar to the iron deposition observed in hereditary hemochromatosis of type I. Liver iron concentration was increased ninefold in comparison with control animals fed standard diet. Animals in the overloaded group also displayed mild hepatomegaly. The iron-loaded liver showed signs of growth stimulation: anisokoria and increased number of hepatocytes with double nuclei and occasional mitoses. Also borderline microvesicular steatosis was observed. We have performed proteomic analysis of liver homogenates of control and iron loaded animals. We reproducibly detected 1020 (± 20) protein spots, out of which 32 displayed changed intensity. We identified 30 of the 32 protein spots by mass spectrometry.

Iron storage

As expected also in this experiment, we observed that the expression of the iron-storage protein ferritin was substantially increased in the iron-loaded livers (L-ferritin 6.7-fold, H-ferritin 3.7-fold). That provides further confirmation of effective liver iron overload in addition to direct liver iron measurement.

Urea cycle

We observed increased levels of three enzymes of the urea cycle (carbamoyl-phosphate synthase, ornithine carbamoyltransferase, and arginase) in iron overload. Urea cycle is an essential five-enzyme system that converts toxic ammonia into urea. A connection of urea cycle with iron metabolism has not been previously established, and the role of urea cycle in iron overload remains to be elucidated. Interestingly, CAAT/enhancer-binding protein (C/EBP alpha), a transcriptional regulator of the genes encoding these enzymes, has been previously shown to be increased by iron excess in mouse liver (Courselaud et al., 2002). C/EBP alpha also regulates expression of hepcidin, a key regulator of intestinal iron absorption, whose levels also increase in iron overload (Courselaud et al., 2002). It is noteworthy that the fifth enzyme of the urea cycle, arginase, competes with nitric oxide synthase for arginine, the sole substrate for nitric oxide production (Scaglia et al., 2004). Nitric oxide is known to play an important role in iron homeostasis by modulating the activity of iron regulatory proteins and non-transferrin iron transport (Hentze et Kuhn, 1996; Richardson et al., 1995). Nitric oxide also alters intracellular iron metabolism through iron release from ferritin (Reif et Simmons, 1990). Conversely, expression of inducible nitric oxide synthase is negatively regulated by iron (Weiss et al., 1994). Nitric oxide may therefore represent

the key link between the urea cycle and iron homeostasis in the liver.

Peroxisomal fatty acid oxidation and sterol metabolism

We noted decreased levels of three enzymes of fatty acid oxidation pathway in iron-overload liver - peroxisomal 2-hydroxyphytanoyl-CoA lyase, peroxisomal enoyl CoA hydratase, and acetyl-CoA thioesterase 1. Decreased levels of these enzymes could indicate impaired fatty acid catabolism in the liver of iron-overloaded mice. Reduced fatty acid oxidation can contribute to the development of liver steatosis (Reddy, 2001), often present in patients with hereditary hemochromatosis. Microvesicular steatosis, although very mild, was present in the liver of iron overloaded mice in our experiment.

Methylation cycle and sarcosine dehydrogenase

Our study also revealed expression changes of two enzymes involved in methylation cycle and methionine metabolism (S-adenosylhomocysteine hydrolase, glycine N'-methyltransferase). Adenosylmethonine (AdoMet)-dependent methylation has been shown to be central to many biological processes including gene regulation via DNA, protein methylation, and biosynthesis of phospholipids. S-adenosylhomocysteine (AdoHcy), that is formed after donation of the activated methyl group of AdoMet to a methyl acceptor is a strong competitor of all AdoMet-dependent methyltransferases.

AdoHcy is removed by hydrolysis via S-adenosylhomocysteine hydrolase (which was decreased in iron overload). Glycine N'-methyltransferase (increased in iron overload) catalyzes the methylation of glycine by using S-adenosylmethionine (AdoMet) to form AdoHcy and sarcosine, a molecule that has no known physiological role in mammals.

Glycine N'-methyltransferase functions to optimize the AdoMet-to-AdoHcy ratio, and consequently the cellular transmethylation potential (Kerr, 1972). Interestingly, sarcosine dehydrogenase, the enzyme that recycles sarcosine back to glycine, was also decreased in iron overload in our study.

We hypothesize that the observed decrease of S-adenosylhomocysteine hydrolase combined with increased level of glycine N'-methyltransferase in iron overload could lead to accumulation of AdoHcy, and consequently to the inhibition of numerous AdoMet-dependent methyltransferases. Decreased activity of these proteins can negatively affect phospholipids synthesis and methylation of proteins, small molecules, DNA, and RNA (Chiang et al., 1996). That could result in a multitude of negative biological effects. Iron-induced alterations of methylation pathway could potentially contribute to the liver damage observed in patients with iron overload diseases.

The expression changes of some other proteins identified in our study (glutathione S-transferase Mu 6, glucose-regulated protein 78/luminal binding protein - GRP78/Bip) reflect more likely a general response of the cells to stress conditions than a specific response to high iron levels (Petrak et al., 2008).

In summary, we identified 30 proteins with altered levels in the liver of mice with nutritional iron overload. The proteins affected give us important clues into the significant metabolic changes in the iron-loaded liver. However, confirmation of the effects and the role of the particular metabolic pathways in molecular pathogenesis of iron overload remains to be determined in future studies.

Results of our study were published in American Journal of Physiology in 2007:

Petrak J, Myslivcova D, Man P, Cmejla R, Cmejlova J, Vyoral D, Elleder M, Vulpe CD. Proteomic analysis of hepatic iron overload in mice suggest dysregulation of urea cycle, impairment of fatty acid oxidation, and changes in the methylation cycle. Am J Physiol Gastrointest Liver Physiol. 2007, 292(6):G1490-8.

4.3. Aim Three:

Are there any liver protein expression changes specific for hereditary hemochromatosis that are not caused by iron overload?

Molecular mechanism of hereditary hemochromatosis type I is unknown, despite the knowledge of underlying mutation in HFE protein. We intended to provide at least partial insight by identification of candidate proteins involved.

This set of experiments was designed to answer the critical question: What changes in protein expression are induced solely by the inactivation of HFE gene and not as a result of increased iron levels. We used a mouse model of hereditary hemochromatosis - mice with defective HFE protein expression (HFE-KO) (Zhou et al., 1998), showing gradual iron overload. To identify the specific changes induced by HFE protein defficiency, we compared HFE-KO mice with a control group of mice without the genetic defect (WT – wild type), both on the same genetic background C57B16/J.

A group of 4-weeks-old male WT mice were fed the standard rodent diet supplemented with 2% carbonyl iron for 8 days to reach hepatic iron concentration comparable with that observed in HFE-KO mice of the same age on a standard diet. The WT mice developed mild nutritional overload and HFE-KO mice were overloaded as a result of HFE deficiency. All changes in protein expression between the groups should therefore be caused by HFE deficiency, and be independent of iron loading.

We analysed liver homogenates of HFE-KO and WT mice by 2-DE. We reproducibly detected 1150 ± 30 spots, out of which 20 showed changed expression. We identified 19 differentially expressed proteins by mass spectrometry. Next we will discuss the proteins with significantly altered expression.

MUPs

Major urinary proteins 1, 2 and 6 (MUPs, up-regulated in HFE-KO) belong to a family of small secreted proteins, lipocalins. Rodent major urinary proteins are synthesized predominantly in liver, released into serum, before being rapidly filtered by the kidney and excreted at very high concentrations in urine. Lipocalins play various roles are known to bind diverse low molecular weight ligands and to perform variety of functions. Interestingly, a member of the family, lipocalin 24p3 (human NGAL-neutrophil gelatinase-associated lipocalin, lipocalin 2) can donate iron to various cells (Yang et al., 2002) via endocytic pathway. More interestingly, 24p3 seems to be also capable of intracellular iron chelation and iron excretion (Ziegler et al., 2007).

We hypothesize, that MUP proteins can take part in iron transport or disposal of oxidatively damaged molecules. Our theory is supported by increased expression of MUPs in studies addressing liver protein expression

elicited by hepatotoxic drugs (Zgoda et al., 2006). However, the actual role of MUPs and other lipocalins in iron metabolism and hemochromatosis remains obscure.

Glutathione-S-transferase P1

Glutathione-S-transferase P1 (up-regulated in HFE-KO) belongs to the GSTs superfamily of enzymes involved in cellular detoxication of toxic and carcinogenic compounds. In addition to its canonic function, glutathione-S-transferase P1 interferes with TNF-alpha signalling by association with TRAF2 (tumor necrosis receptor-associated factor 2) (Wu et al., 2006). Tumor necrosis factor α (TNF-α) is a cytokine with diverse functions which contributes to the regulation of iron metabolism by inhibiting intestinal iron absorption and iron release from peritoneal macrophages (Laftah et al., 2006) while also stimulating synthesis of ferritin and transferrin receptor (Alvarez et al., 1989). Based on the involvement of GST-P1 in the TNF-alpha signalling, it could be hypothesized, that GST-P1 (induced by HFE disruption) in the HFE-KO mice can influence iron metabolism by abrogating TNF-alpha signalling.

Selenium binding protein 2

The biological function of selenium binding protein 2 (SBP2) is not known. It is expressed predominantly in liver and represents a major target for acetaminophen toxicity (Pumford, et al., 1992). However, recently it was suggested that SBP2 is implicated in liver fibrosis (Henkel et al., 2005).

It is of interest, that expression of SBP2 in liver is reduced under peroxisome proliferator-activated receptor alpha (PPAR alpha) activation (Chu et al., 2004) and this PPAR alpha-related down-regulation is

accompanied by decreased expression of glutathione-S-transferase P1 and major urinary protein 2 (Chu et al., 2004).

In our current study we observed an opposite regulation pattern – up-regulation – of these three proteins in the HFE-KO. This suggests that in HFE-KO mice we are witnessing a process adverse to the PPAR alpha activation.

Carboxylesterase 1

Liver carboxylesterase 1 (CE1) is a broad spectrum serine esterase (Redinbo et al., 2003). The normal physiologic roles of the human ortholog hCE1 are in cholesterol and fatty acid metabolism. The human CE1 plays also an important role in xenobiotic metabolism and certain pro-drugs (for instance lovastatin - cholesterol lowering compound) are processed to their active forms by this enzyme. The down-regulation of liver carboxylase 1 (if confirmed in patients with herediatry hemochromatosis type I) would be of utmost clinical significance given the role of hCE1 in drug metabolism.

We identified a HFE hemochromatosis-specific protein expression pattern in the liver of HFE-KO mice. The pattern is not secondary to increased hepatic iron, but result directly from the HFE inactivation. Therefore, we hypothesize that the proteins differentially expressed in HFE-KO animals are directly or indirectly involved in the molecular pathology of hereditary hemochromatosis type I. The particular roles of the differentially expressed proteins in hemochromatosis are yet to be elucidated. However, our data allows us to propose an involvement of lipocalins, and PPAR alpha regulatory pathway in the pathogenesis of hereditary hemochromatosis.

The results of our study were published in The International Journal of Biochemistry & Cell Biology in 2007:

Petrak J, Myslivcova D, Halada P, Cmejla R, Cmejlova J, Vyoral D, Vulpe CD. Iron-independent specific protein expression pattern in the liver of HFE-deficient mice. Int J Biochem Cell Biol. 2007;39(5):1006-15.

5. Conclusions

In our three presented analyses we identified three distinct sets of proteins involved in 1) response of liver (hepatoma) cells to acute iron toxicity, 2) response of liver to chronic nutritional iron overload and 3) pathophysiological mechanisms of hereditary hemochromatosis type I.

The results of our study have provided us with new information that will serve as a basis for future detailed studies addressing particular roles of individual candidate proteins or pathways in the context of iron metabolism. Such studies are prerequisite for detailed understanding of molecular mechanisms involved in iron metabolism. Without the understanding of molecular pathophysiology it is impossible to imagine any progress in the development of new methods in prevention and therapy of diseases connected with iron metabolism, namely hemochromatosis and anemia from chronic diseases.

6. References

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7. List of publications related to the thesis

Petrak J, Myslivcova D, Man P, Cmejla R, Cmejlova J, Vyoral D.
Proteomic analysis of iron overload in human hepatoma cells. *Am J Physiol Gastrointest Liver Physiol.* 2006 May;290(5):G1059-66.
Impact factor: 3,472

Petrak J, Myslivcova D, Man P, Cmejla R, Cmejlova J, Vyoral D, Elleder M, Vulpe CD.
Proteomic analysis of hepatic iron overload in mice suggests dysregulation of urea cycle, impairment of fatty acid oxidation, and changes in the methylation cycle. *Am J Physiol Gastrointest Liver Physiol*. 2007 Jun;292(6):G1490-8.
Impact factor: 3,681

Petrak J, Myslivcova D, Halada P, Cmejla R, Cmejlova J, Vyoral D, Vulpe CD.
Iron-independent specific protein expression pattern in the liver of HFE-deficient mice. *Int J Biochem Cell Biol*. 2007;39(5):1006-15.
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